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13. ABSTRACT *(Maximum 200 words)*

The **human estrogen receptor** (hER), is the *in vivo* target of the female sex hormone estrogen. It is also the therapeutic target of various **antiestrogens** used during hormonal treatment in the battle of breast cancer. The purpose of this research is to exploit the known **structure-function** relations of the hER protein to develop novel approaches at regulating hER function. The scope of this research involves the study of novel **inhibitory peptides** which appear to block hER **dimerization.** Our latest findings have compelled a revision of our earlier hypothesis regarding the **phosphotyrosine** mediated dimerization of the hER. During the last year we have made significant progress in understanding the role of tyrosine 537 in hER function and in evaluating phosphopeptides as hER inhibitors. We now show that the phosphotyrosyl peptide, derived from the C-terminal helix 12 of the hER ligand binding domain (LBD), inhibits hER DNA binding through a LBD -dependent mechanism. The sequence specificity of this peptide is also further characterized.

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Mark R. Hoff 8/11/98

PI - Signature Date

Table of Contents

INTRODUCTION

The human estrogen receptor (hER) is a member of the steroid/thyroid hormone receptor superfamily of ligand activated transcription factors. The hER functions as a homodimer which binds specific DNA response elements (EREs) in the promoter regions of estrogen responsive genes. The molecular mechanisms by which these receptors regulate transcription is an area of considerable research focused on diverse areas including protein-protein interactions with transcriptional intermediaries. The recently solved three dimensional structure of the hER ligand binding domain (LBD) has lead to a greater understanding and interest in developing drugs targeting the estrogen binding site. The overall research objectives described here are aimed at exploiting known structure-function relations to develop novel approaches at regulating hER function.

Previous published reports from this laboratory have proposed a phosphotyrosine mediated dimerization mechanism for the hER ([1], [2]). In this model, tyrosine 537 (Y537) phosphorylation of one monomer interacts with another tyrosine phosphorylated monomer to constitute an hER dimer. Supporting this hypothesis is the important observation that a 12-amino acid phosphotyrosine peptide, derived from the wild type protein sequence surrounding Y537, is capable of blocking receptor dimerization.

The purpose of this research is to identify, isolate and structurally characterize the putative phosphotyrosine (pY) binding site of the hER which is believed to be the molecular basis of the coupling between

monomers.

The specific aims of this project are,

1. Use molecular and biochemical approaches to identify and isolate the minimal region of the hER sufficient for phosphotyrosyl mediated dimerization.
2. Develop a yeast two-hybrid system to characterize the *in vivo* function of the pY binding domain and dimerization deficient mutants.
3. Determine the recognition specificity of the pY-pY binding domain using phosphotyrosyl peptides and mutagenesis of the domain.
4. Structurally characterize the pY binding domain and peptide interactions using nuclear magnetic resonance (NMR).

Background of previous work

The development of the phosphotyrosine mediated hER dimerization hypothesis is documented ([1-4]). In the first year of this project, progress was made in determining a phosphopeptide binding region within the hER. The region between amino acids 251-340 was found to specifically bind the phosphopeptide, Yp-12 (figure 1). Further progress on the specificity and sequence requirements of the peptide itself was also made in year one. It was found that in addition to the phosphotyrosine, amino acids on the carboxy terminal (Yp-7C, figure 1) end of the peptide were also determinants for hER-phosphopeptide interactions. Data was also presented suggesting amino acids at the +1 and +3 sites, relative to the phosphotyrosine, where critical for peptide function.

BODY

The body of this report will discuss the results related to the specific tasks as outlined below in the original Statement of Work (SOW). Each technical objective corresponds to a specific aim (previous page) of the proposal. The results presented on the following pages are organized for each specific aim and refer to these objectives and tasks.

Statement of Work

Technical Objective 1.

By the end of month 2 have identified the phosphotyrosine binding (PTB) domain of the hER and expressed in bacterial system

Technical Objective 2.

Task 1. Months 3-6: Construction of yeast strains and development of vectors for two-hybrid system.

Task 2. Months 7,8: Characterization PTB domain utilizing two-hybrid system.

Technical Objective 3.

Task 1. Months 11,12: Develop in-vitro assay to quantify peptide affinities/specificities.

Task 2. Months 13-15: Determine specificity and affinity of PTB domain for peptide ligands

Task 3. Months 16-20: Mutational analysis of PTB domain.

Technical Objective 4.

Task 1. Months 21,22: Overexpress, solubilize and purify hER PTB domain

Task 2. Months 23-30: Determine high resolution, atomic scale image of hER PTB by NMR.

Task 3. Months 31-36: NMR analysis of peptide/PTB interaction.

Month 37 Write Ph.D thesis

Month 38 Defend Ph.D thesis

Results and Discussion

Aim 1. Identification of phosphopeptide binding site on the hER.

Technical Objectives

By the end of month 2 have identified the phosphotyrosine binding (PTB) domain of the hER and expressed in bacterial system

Last years report described the identification of the hER 'hinge' or D domain, as a binding site for the phosphopeptide, Yp12 (figure 1a). Using far western and affinity 'pull-down' techniques, this region was found to contain a binding specificity. However, attempts of using immobilized peptide affinity chromatography to study this interaction were unsuccessful. Despite its extreme insolubility, a collaboration with Dr. Shohei Koide resulted in a solubilization protocol and analysis by NMR. These results indicate an unstructured protein both in the absence and presence of the phosphopeptide (Ref. Dr. Shohei Koide, unpublished results, personal communication).

In light of these results, further approaches were designed to test the possibility of experimental artifact in the identification of the hinge region as the peptide binding site. These experiments were designed to gain further insight into a possible peptide binding mechanism in addition to confirming the location of a phosphopeptide binding site. **The tested hypotheses are outlined below:**

1. Removal of cysteine to prevent sulfhydryl oxidation in peptide-hER interactions.

As a first step, the Yp-12 phosphotyrosine peptide was shortened to a Yp-11 phosphopeptide by the exclusion of the N-terminal cysteine residue (figure 1b). The purpose of this control is to eliminate the possibility of sulfhydryl oxidation reactions in the interaction with the hER. Since it was shown in last years report that the C-terminal residues of the Yp-12 peptide were the critical determinants, the removal of the N-terminal residue was not expected to alter phosphopeptide specificity. Gel mobility shift assays confirm that this change does not affect peptide function. The following experiments were carried out with the Yp11 phosphotyrosine peptide.

2. Competitive inhibition of hER binding to an estrogen response element (ERE).

Although previous data suggests otherwise ([1]), we tested the peptide in gel shift assays as a competitive inhibitor of ERE binding. From Michaelis-Menton kinetics we know that a competitive inhibitor is one which will shift a saturation curve to the right, indicating an affect on Km, while a non competitive inhibitor reduces maximum binding at all concentrations. With a simple gel shift assay, using a constant amount of recombinant hER and increasing concentrations of 32P-labeled ERE, a saturation curve was generated for hER DNA binding in the absence and presence of 100 μM Yp-11. Over the range selected, no differences in DNA binding inhibition were observed, indicating a non-competitive inhibition (figure 2). These data suggest that the mechanism of phosphopeptide competition does not involve the DNA binding domain or competition with the ERE.

3. Phosphopeptide hormone binding competition

The hER antagonists, ICI 164,384 and ICI 182,780 are competitive inhibitors of the natural estrogenic hER agonists ([5]). It is also known that under some conditions, the ICI compounds will inhibit DNA binding in gel shift assays. This phenomena has been attributed to receptor turnover *in vivo* ([6]), and with blocking dimerization *in vitro* ([7]). The phosphopeptide was tested side-by-side with ICI 182,780 in hormone binding competition assay using [3H]-estradiol. As seen in figure 3, the ICI compound competes with the labeled hormone for binding to the ligand binding domain. In contrast, the phosphopeptide has no affect on bound [3H] estradiol at concentrations in excess of those needed to block DNA binding. These data suggest that the phosphopeptide does not bind to the ligand binding pocket and the mechanism for DNA binding inhibition is different than observed for the antiestrogen ICI 182,780.

4. Inactivation from aggregation or degradation.

A simple western blot was used to measure any changes in receptor concentration and degradation when incubated with the phosphopeptide. Following a 24 hour incubation of hER containing extracts under DNA and hormone binding assay conditions (see methods and procedures), no effect of peptide was observed (figure 4). Aggregation or precipitation was tested in these assays by performing a 15 minute centrifugation to remove insoluble protein aggregates. Again, no differences were observed in soluble hER in the presence of phosphopeptide.

Phosphopeptide Inhibition Requires the Ligand Binding Domain.(LBD)

To identify specific regions of the hER necessary for phosphopeptide interaction, in vitro translated fragments of the hER were prepared. The two fragments are hER A-D, from amino acids 1-340, and hER C-F, amino acids 330-595. Both fragments contain an intact DNA binding domain and the hinge region. They specifically bind to EREs in gel shift assays as seen with antibody super shifts assays (figure 5, lanes 2&3 and personal communication with G. Sathya). The phosphopeptide inhibits DNA binding of the full length wt *in vitro* translated hER and the C-F hER, but has no affect on the A-D hER fragment (figure 5). The phosphopeptide requires the presence the LBD for inhibition of DNA binding. It is likely that the peptide binds directly this region, but our data do not show this region alone binds the peptide. It is conceivable that hinge region amino acids, and a DNA binding dimeric conformation are required. The GST-hER LBD fusion protein described in last years report (Yearly Report 1997) is extremely insoluble and we were not able to generate an active soluble LBD to test this hypothesis. Future studies may involve preparation of the LBD as prepared for the X-ray crystallography analysis by Brozowski et. al. ([8]).

Recommendations in relation to SOW

Aim 1. This aim is complete. The region of phosphopeptide interaction has been localized to the LBD. Although the precise mechanism is unknown, experiments described under Aim 4 will address the question of mechanism.

Aim 2: Analysis of Phosphotyrosine Mediated hER Dimerization in Yeast

Task 1. Months 3-6: Construction of yeast strains and development of vectors for two-hybrid system.

Task 2. Months 7,8: Characterization PTB domain utilizing two-hybrid system.

As previously stated, the overall objective of this project is to characterize the phosphotyrosine mediated dimerization of the hER. The goal of this aim is to use a rationally designed yeast two hybrid system to characterize hER dimerization and the role of tyrosine 537. The development of the two-hybrid assay was preluded with the generation of a 'one hybrid' or simple yeast expression report system. This basic system was designed to characterize the function of hER in yeast and to establish the effects of the tyrosine to phenylalanine mutation in a cellular model system. This mutation has been hypothesized to yield a 'dimer deficient' hER. The results of these experiments as described below refute this hypothesis and suggest tyrosine phosphorylation at this site is not essential for hER function, and therefore receptor dimerization.

The yeast system was developed from an existing protocol developed in our laboratory ([9]). The mutations Y537F, L540Q, and the double mutant (dm), Y537F/L540Q were generated by Dr. Daria Vorojeikina, cloned into expression vectors and transformed into the yeast reporter containing yeast strain. The Y537F mutation will test the role of this site in hER function. The L540Q hER is a dominant negative receptor, and we now know is part of the AF-2 binding site for co-activators ([10]). The generation of this mutant hER and its combination with Y537F in the double mutant (dm), Y537F/L540Q, was designed to both test and distinguish the significance of each site in relation to each other.

Figure 6 illustrates the results of the indicated hER mutations on the transactivation potential in yeast. The Y537F results in only roughly 20%

decrease in activation. This affect, albeit small, is additive with the hER L540Q mutation. The 50% transactivation decrease observed for the L540Q is further potentiated in the double mutant. These results are apparently not due to differences in expression level as indicated by a western blot on yeast extracts with an anti-hER antibody (figure 6b). The yeast expressing wt and mutant hERs were tested for ligand binding specificity and were found to function similarly (figure 6c). These results indicate that Y537F and L540Q mutations affect the transactivation capacity of the hER and are not a result in differential hormone binding properties in yeast.

Recommendations in relation to SOW

Aim 2. This aim is complete. The yeast expression/reporter system designed to test the role Y537 has led to the re-evaluation of the initial hypothesis regarding the role of tyrosine phosphorylation in hER dimerization. Y537F is not a dimer deficient hER. Furthermore, phosphorylation of Y537 is not required for transactivation in yeast. The hypothesis for which the yeast two hybrid system was developed to test, and as initially described in this proposal, has been falsified. This objective cannot proceed as initially described, and no further experiments in yeast are planned.

However, we are currently developing a cell-based assay to study the effects of peptide hER inhibitors. In these experiments, we are attempting to introduce peptides into cultured cells expressing endogenous (MCF-7 cells) or recombinant (COS-7 cells) hER. It is felt that this cell-based approach to study the inhibitory peptides described in this report fits with the objectives outlined for this specific aim.

Aim 3. Biochemical analysis of phosphopeptides and hER helix 12 mutants.

Task 1. *Months 11,12: Develop in-vitro assay to quantify peptide affinities/specificities.*

Task 2. *Months 13-15: Determine specificities and affinities of PTB domain for peptide ligands*

Task 3. *Months 16-20: Mutational analysis of PTB domain.*

Phosphopeptides

A detailed analysis of the phosphopeptide specificity in hER interaction and DNA binding inhibition was given in last years report. To summarize,

1. The specificity for phosphotyrosine was established
2 The phosphopeptide bound directly to the hER, not through another factor.
3. The residues carboxy terminal to phosphotyrosine (Yp-7C) were found to be important for maximal peptide activity.

Additional specificity and sequence requirements were analyzed since the last report. Figure 7 shows the result of several new peptides designed to test the specificity of the hER interaction. Changing the pY to a serine, or alanine (fig. 1b) abolished inhibitory capacity. Serine and alanine are not functional replacements for the phosphotyrosine in this peptide. These changes were made in light of the constitutively active hER mutations Y537S and Y537A ([11]). A longer peptide, pY-16 (fig. 1b), designed to include the entire helix 12 sequence was also synthesized. This peptide had a similar activity to that of the shorter 11 amino acid

peptide, indicating the longer peptide is not a better inhibitor. Another test for sequence specificity was carried out by simply changing the position of the phosphotyrosine in relation to the 11 residue sequence. When shifted 4 residues towards the carboxy terminal end, the peptide is inactive, another indication for sequence and structural specificity of pY-11 hER interaction.

hER mutations

A detailed biochemical analysis of the mutations studied in the yeast transcription assay (under **Aim 2**) were carried out. The DNA and hormone binding affinities were measured, as well as an estimation of the Hill coefficient for estradiol binding.

The Y537F, L540Q and dm mutant hERs were cloned into a baculovirus expression system as previously described in this laboratory for the wt hER ([12]). Using cell extracts and ammonium sulfate precipitated fractions, the hormone binding properties of the four hERs were compared. No significant differences were seen for estradiol affinity or hill coefficient at various concentrations (fig 8). These results suggest that these sites, or these mutations, do not affect hormone binding to the hER. The differences in yeast transactivation are not caused by different hormone binding properties.

The same preparations were analyzed on western blots to accurately determine relative concentrations of total hER protein. Using equivalent amounts of hERs, the DNA binding properties were analyzed and compared to wt. Again, under the conditions tested, no significant difference in ERE binding affinities were observed (fig. 9). However, when testing DNA binding properties in the absence and presence of estradiol, an interesting

difference between the wt and Y537F hER was discovered. The wt hER, when incubated overnight on ice with 100 nM estradiol results in a stabilizing effect not observed with the Y537F mutant. A gel shift saturation binding assay of these overnight incubations display an increase in the maximum binding, or capacity, of the hER for ^{32}P-labeled ERE. This increase in B_{max} is not seen with the Y537F when incubated with estradiol under identical conditions. This result can be interpreted as a loss of estradiol induced hER stability when Y537 is changed to phenylalanine. This hypothesis is rational in light of the structure and function of this region of the ligand binding domain. Furthermore, the reports of Katzenellenbogen ([11], [13]) and Parker ([14]) on constitutive activation observed with other Y537 mutants indicate a stabilization of a 'closed' confirmation in these mutants independent of bound hormone. It is conceivable that the Y537F mutation has a slightly opposite affect, de stabilizing the helix 12 confirmation and favoring an 'open' and less stable protein, in the absence or presence of estradiol. Figure 10 depicts a model illustrating the role of specific mutations studied by us and others and their proposed affects on stability and/or coactivator binding functions of the hER.

Recommendations in relation to SOW

Aim 3. This aim is complete. The sequence specificity of the phosphopeptides has been thoroughly studied. The second objective within this aim is the 'mutational analysis of the peptide binding domain' has been modified to a mutational analysis of Y537 and helix 12. Presented in this report is a detailed analysis of two hER mutations, Y537F and L540Q. These studies have led to important conclusions regarding the function of hER tyrosine 537, and the to the development of a model for

this and other helix 12 mutations.

Aim 4. NMR structural analysis of hER peptide interactions

Task 1. *Months 21,22: Overexpress, solubilize and purify hER PTB domain*

Task 2. *Months 23-30: Determine high resolution, atomic scale image of hER PTB by NMR.*

Task 3. *Months 31-36: NMR analysis of peptide/PTB interaction.*

This aim is focused on a collaborative effort with Dr. Shohei Koide to analyze the phosphopeptide-hER interactions using nuclear magnetic resonance (NMR) spectroscopy.

Last years report indicated the 'hinge' or D domain of the hER as the location of phosphopeptide binding interactions. This protein fragment was expressed as a histidine-tag fusion protein, solubilized, and cleaved from the GST fusion with thrombin. The resulting pure hER hinge domain fragment was analyzed by NMR in the absence and presence of the phosphopeptide. In both cases, with and without peptide, the protein fragment appeared to lack any structural features commonly associated with stably folded proteins in solution. The lack of structure and peptide effects seem to indicate the original identification of this region as a phosphopeptide binding site as a non-specific artifact. This conclusion is supported by the work illustrated under **Aim 1** above, indicating the ligand binding domain (LBD) is necessary for peptide-hER interactions

Since last years report, the structure of the hER LBD has been solved by X-ray crystallography in two independent reports ([8],). It does not appear to contain a phosphopeptide binding site. However, the function of helix 12, from which the phosphopeptide is derived, is quintessential to

the function of the whole protein. We are currently developing experimental approaches to understand the mechanism of phosphopeptide-hER interaction.

A Dimer Contact Inhibitor Peptide.

An analysis of the phosphopeptide sequence, which is derived from helix 12, shows a homology with the dimerization contact region of helix 11 (see figure 1a). To test the hypothesis that the function of phosphopeptides is a mimicry of the now known dimerization contact site of this helix ([8]), a new 'dimer contact' peptide sequence is being tested. The hypothesis is that a peptide containing the correct dimer contact sequence and structural features will inhibit dimerization through a competitive mechanism. Preliminary data supports this hypothesis and are under investigation at this time.

The following hypotheses are being tested regarding a peptide hER inhibitory mechanism:

a.) The Yp11 peptide mimics helix 12 and interacts with the LBD surface where this helix lies in holo-hER.

b.) The Yp11 peptide mimics helix 11 which is the major dimer interface observed in the crystal structure. The phosphopeptide is homologous to this region.

c.) The Yp11 peptide works through some other mechanism not yet described.

Recommendations in relation to SOW

Aim 4. This aim is incomplete. Although the structure of the LBD (and putative peptide binding region) is known, the objective of this aim is to understand the mechanism of peptide-hER interactions. Future studies

involve obtaining a soluble ligand binding domain to test the following hypotheses using biophysical approaches:

CONCLUSIONS

Aim 1

1. The phosphopeptide - hinge domain interactions are a possible artifact of the E. coli produced proteins. Since this region, shown to contain a weak affinity for the phosphopeptide, is unstructured in both the absence or presence of the peptide, it is not known to be relevant to the functional receptor.
2. Phosphopeptide inhibition requires the presence of the ligand binding domain (LBD) in a functional assay.
3. The phosphopeptide does not compete for or prevent estradiol binding. The phosphopeptide is a non-competitive inhibitor of DNA binding, as saturating amounts of ERE do not compete for binding to hER. The peptide does not precipitate or aggregate the hER, or cause a degradation of the receptor.

Aim 2

1. Y537F hER is NOT a dimerization deficient mutant. This negative data compels us to re-interpret previous findings and conclude that tyrosine 537 phosphorylation is not essential for function of the hER.
2. The Y537F transactivation effects are additive in combination with a second mutation, L540Q.

Aim 3.

1. Peptide inhibitors requires phosphotyrosine, the amino acids immediately C-terminal, and the correct location of the phosphotyrosine within the sequence

2. Y537F hER from Sf9 is functional *in-vitro* in both hormone and DNA binding properties.

3. Y537F has wild type affinity for estradiol and the estrogen response element

4. Y537F appears less stable than wild type hER.

EXPERIMENTAL METHODS AND PROCEDURES

Materials

The 3H Estradiol (50 Ci/mmol) and 32P-g-ATP were purchased from Dupont-NEN (Boston, MA). The ICI 178, 820 was kindly provided by Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Mereside, UK). Tamoxifen citrate was obtained from Stuart Pharmaceuticals (Wilmington, DE). Glass beads, 0.5 mm in diameter, for breaking yeast cell walls, were purchased from Biospec (Bartesville, OK). The Bio-Rad ___ kit was used for site-directed mutagenesis and the dideoxy sequenase kit from USB was used for sequencing. Anti-hER polyclonal antibody 6, directed against amino acids 259-278 (?) was affinity purified and used in western blotting. Anti-hER polyclonal antibody 5, directed against amino acids 150-165 (?) was affinity purified and used in gel supershift assays. Phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology, Lake Placid, NY. All secondary antibodies were purchased from Santa Cruz Biotech.

Site-directed mutagenesis

Oligo-directed mutagenesis of the hER was performed by the method of Kunkel. The hER cDNA (HEGO) was cloned into M13mp19 to produce single stranded DNA. Oligonucleotides used for mutagenesis were: Oligo #1-Y537F-.....; Oligo #2-L540Q-..... Y537F mutated DNA was used for the second round of mutagenesis together with oligo #2 to synthesize the double mutant Y537F/L540Q. All mutations were verified by DNA sequencing. Mutated hER cDNA were cloned into the EcoR1 site of both the yeast expression vector pSCW231([9]) and the baculovirus transfer vector pVL1393 (Invitrogen, San Diego CA). The orientation of cloned inserts were verified by restriction enzyme digests with BglII and SmaI.

Yeast strains, growth conditions and transfections.

The Saccharomyces cerevisiae yeast strain 939 was used was used for all experiments ([15]). Yeast cells were grown in minimal yeast medium (0.67% [w/v] yeast nitrogen base without amino acids and 2%

[w/v] glucose) supplemented with the required amino acids. Yeast were transformed by the lithium acetate procedure ([16]). The cells were then plated on minimal yeast medium supplemented with leucine (100 mg/ml). In these transformations, plasmid DNA consisted of a 1:2 mixture of the yeast expression vector pSCW231-hER or appropriate mutant hER and the reporter plasmid YRPE2 ([17]). This plasmid contains two copies of a consensus estrogen responsive element upstream of the cyc promoter linked to a lac Z reporter gene. Successfully transformed yeast cells were able to restore auxotrophy and grow on synthetic glucose minimal plates without uracil and tryptophan (SD/-ura-trp).

Quantitative β galactosidase assays

Yeast cells from a single colony were grown overnight at 30 C to an OD_{600} of 1.0 - 1.5. Following dilution to an OD_{600} of 0.1, 2 ml aliquots were transferred to 18 mm diameter glass tubes where 20 µl of the appropriate ligand solution was added. Incubation was continued in an orbital shaker at 250 rpm for 16 hours. The ligand treated cells were collected by centrifugation and b-galactosidase activity was assayed using whole, permeabilized cells. The values were expressed in Miller units according to the following equation:

$$\text{Miller units} = (1000 * OD_{420})/(t * V * OD_{600})$$

where OD_{420} is the optical density of the o-nitrophenol solution at 420 nM; t is the elapsed time of incubation with the ONPG substrate, in minutes; V is the volume of the reaction in milliliters; and OD600 is the optical density of the cells.

Preparation of recombinant hERs

Spodptera frugipedra (Sf9) cells were infected with baculovirus containing wt or the mutant hER cDNA, grown for 4 days at 27 C, and lysed by repeated freeze/thaw cycles in a hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na3VO4, 0.5 mM Pefa block, and 80 µM leupeptin, pH 7.4). KCl was added to a final concentration of 500 mM and extracts were incubated on ice for 30 minutes before centrifugation at 28,000 X g. Supernatants were either used as soluble cell extracts, or the hER partially purified by 40% ammonium sulfate precipitation.

Electrophoretic mobility shift assays

Whole cell extracts or ammonium sulfate fractions of Sf9 produced hER were prepared as described above. The gel mobility experiments were performed by incubation of equivalent amounts (approx. 6 nM) of wt or

mutant receptor extracts in binding buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 0.2 mM Na3VO4) with 1 mM leupeptin and 0.5 mM Pefabloc (Boehringer Mannheim). The final salt concentration was maintained at 100 mM by appropriate addition of 4M stock solution. Similarly, total protein concentrations of 0.2 mg/ml were achieved using 10 mg/ml insulin. Non specific DNA binding was prevented by pre-incubation of 1 ug of poly(dI-dC) (Pharmacia Biotech.) for 10-15 min. A double stranded 27-base pair probe was 32P-labeled and added to the final reaction (1-10 fmoles = 20,000 cpm). Following a one hour incubation on ice, the samples were electrophoresed on a 5% non-denaturing polyacrylamide gel for 2.5 hours, 175 V, 4 C, in a 0.5X TBE running buffer. The gels were dried and exposed to PhosphorImager plates. Band intensities were quantified using ImageQuant software (Molecular Dynamics) and saturation plots created and analyzed using Sigma Plot.

Hormone binding assays

Ammonium sulfate precipitated hERs were used with final hER concentrations of 2 or 10 nM in a TDEE buffer (40 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM Pefa block, 0.5 mM leupeptin, 10% glycerol, and 150 mM KCl). Gamma globulin was added to achieve a final protein concentration of 5 mg/ml. For saturation binding experiments the receptor preparation was incubated with various concentrations of [^{3}H]estradiol (0.3 - 100 nM). Non-specific binding was measured by a parallel incubation with a 200-fold excess of unlabeled estradiol. Equilibrium was achieved by incubation for 16 hr. on ice, and an aliquot of each mixture was removed to determine the total [^{3}H]estradiol concentration. The unbound hormone was removed by incubation with dextran-coated charcoal solution (0.03%/0.3% final concentrations) for 10 minutes at 0 C with periodic re suspension. Following centrifugation for 5 min. to remove the charcoal, the bound hormone was determined by liquid scintillation counting of the supernatant.

Western blots

Protein extracts from yeast were prepared by the glass bead method according to Kaiser et. al ([18]). Extracts (Sf9 or yeast) were loaded onto a 10% acrylamide gel and resolved by electrophoresis at 30 mA for 6 hour. The proteins were transferred to PVDF membrane at 20 V for 12 hr in a Tris-glycine buffer containing 15% methanol and 0.025% SDS. The membranes were washed in TBST (20 mM Tris, pH 7.4, 0.5 mM NaCl, 0.1% Tween-20) and blocked in the same buffer containing 10% non-fat milk for 1 hour. For detection of the hER, the membranes was probed with affinity

purified antibody 6 in 10% milk TBST for 2 hours at room temperature. After washing, the membrane was re blocked for 30 min and then probed with a 1/2000 dilution of HRP conjugated anti-rabbit IgG for 1.5 hrs. Following extensive washing, hER was detected using the ECL kit (Amersham).

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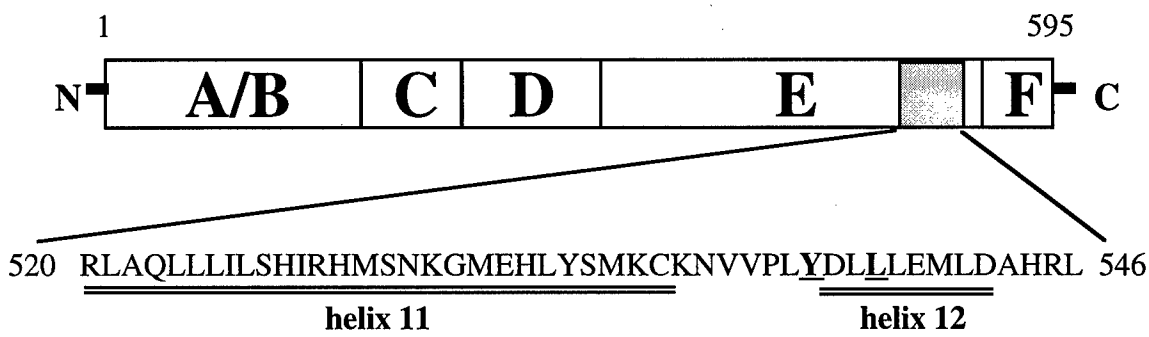
Figure 1.

A



B

Yp-11	N V V P L Y D L L L E
Y-11	N V V P L Yp D L L L E
Yp-7C	L Yp D L L L E
Yp-7N	N V V P L Yp D
Yp-16	N V V P L Y D L L L E M L D A
Ala-11	N V V P L A D L L L E
Ser-11	N V V P L S D L L L E
Yp-11scrmbld	N V V P L D L L L Yp E

C

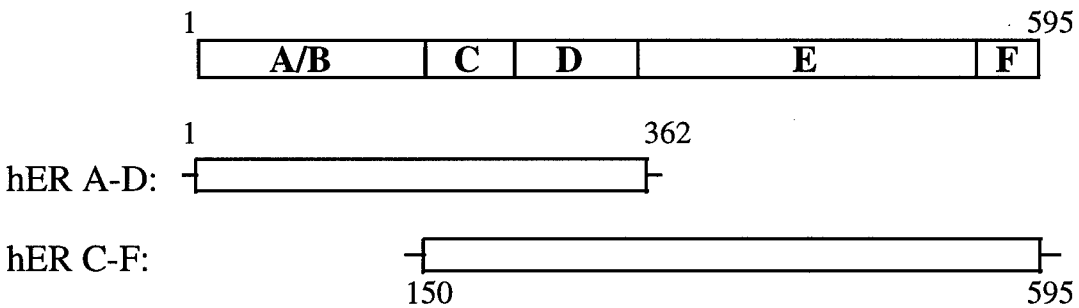


Figure 1. Domains, sequences and peptides referred to in this report.
A) Domains of the hER with the sequence of the carboxy terminal end of the hER ligand binding shown. The demarcation of the last two helices (11 and 12) is shown. Tyrosine 537 and leucine 540 are indicated in tall bold letters. B) Peptides used in this report. The phosphopeptide Yp11 and all of the controls referred to in the text are illustrated. The phosphate is always indicated as a p. C) The two fragments used in figure 5 are shown in relation ot the full length protein.

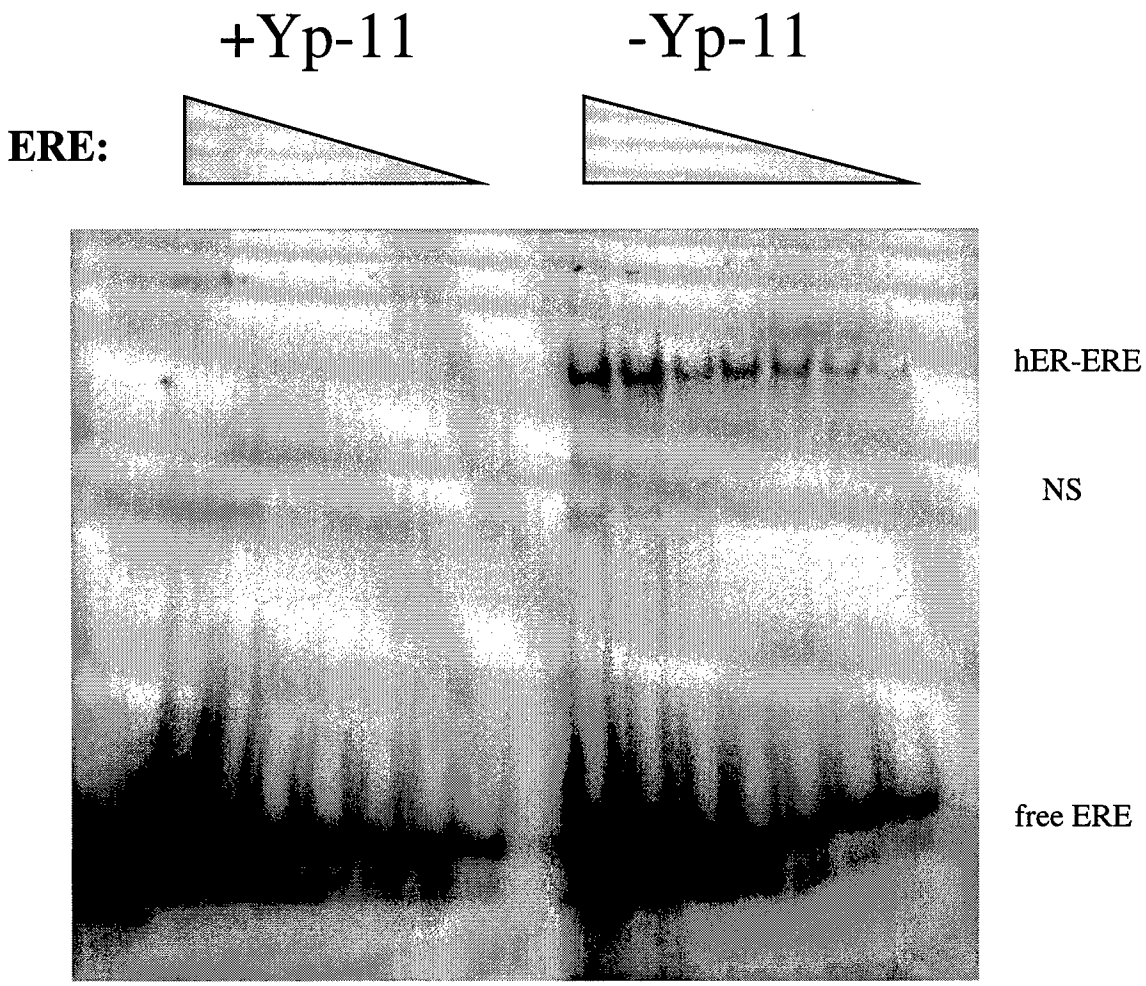


Figure 2. Non-competitive inhibition of DNA binding by phosphopeptide Yp-11.

A gel shift assay showing a non-competitive inhibitory mechanism for peptide inhibition of hER ERE binding. A constant amount of hER containing Sf9 cell extracts were used in each lane with increasing concentrations of 32P-labeled ERE as indicated by the triangles. The presence or absence of 100 μM Yp-11 peptide is shown above the triangles. The specific hER-ERE band is shown along with a non specific (NS) band and the free ERE at the bottom of the gel.

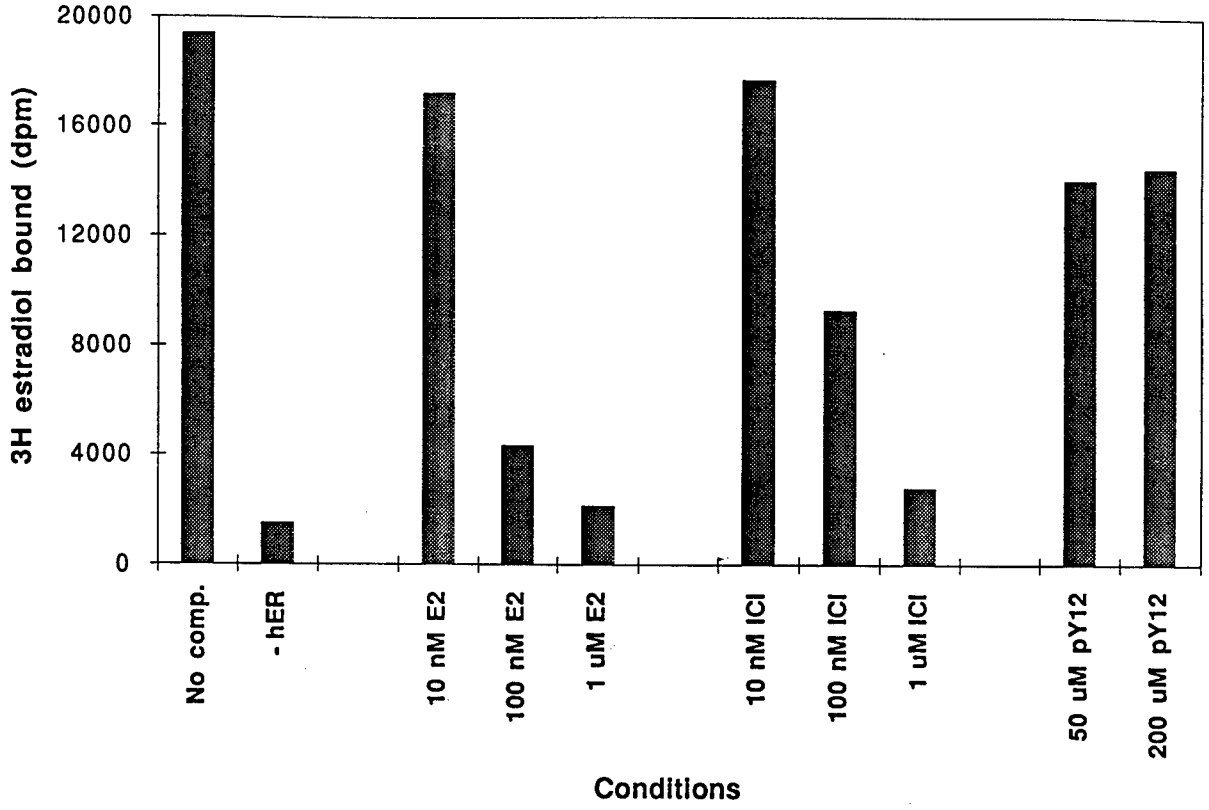


Figure 3. Estradiol competition assay: ICI vs Yp-11

Competition binding assay for [3H] estradiol to the wt hER. The first two bars indicate total and non specific binding, respectively. Both unlabeled estradiol and ICI 182,780 (10 nM, 100 nM, and 1 μM each) can compete for binding to hER. The Yp11 peptide does not compete with [3H] estradiol at 50 or 200 μM.

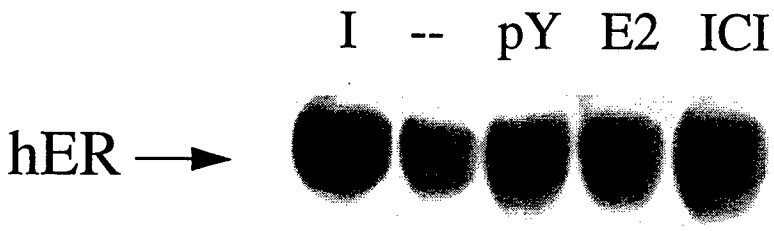


Figure 4. Phosphopeptide does not precipitate or render the hER more susceptible to proteolysis.

A western blot against hER incubated overnight with various ligands and peptide. I = Input protein (no overnight incubation); pY = 100 μM Yp-11 peptide; E2 = 100 nM estradiol; ICI = 100 nM ICI 182,780; -- indicates overnight incubation in absence of ligand or peptide. Equivalent amounts of receptors were centrifuged following overnight incubation, to remove debris and precipitated proteins.

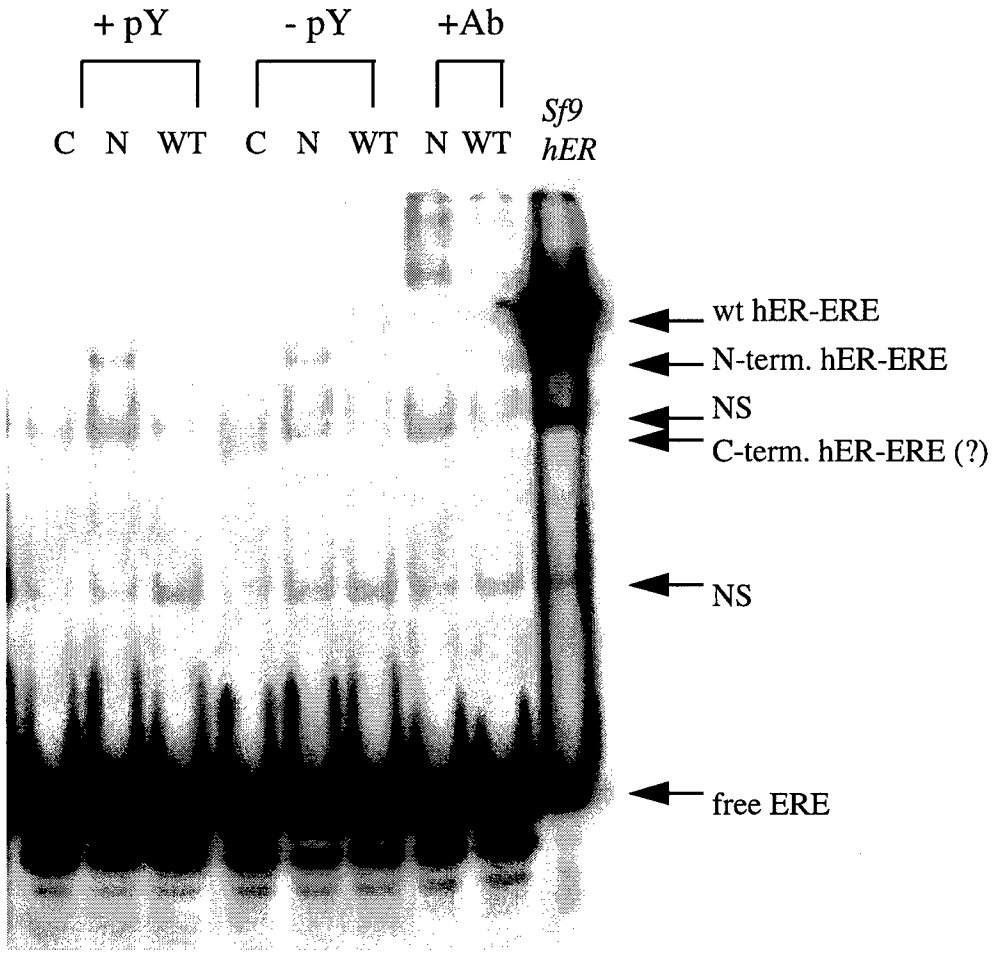


Figure 5. The phosphopeptide inhibition of hER ERE binding requires the ligand binding domain.

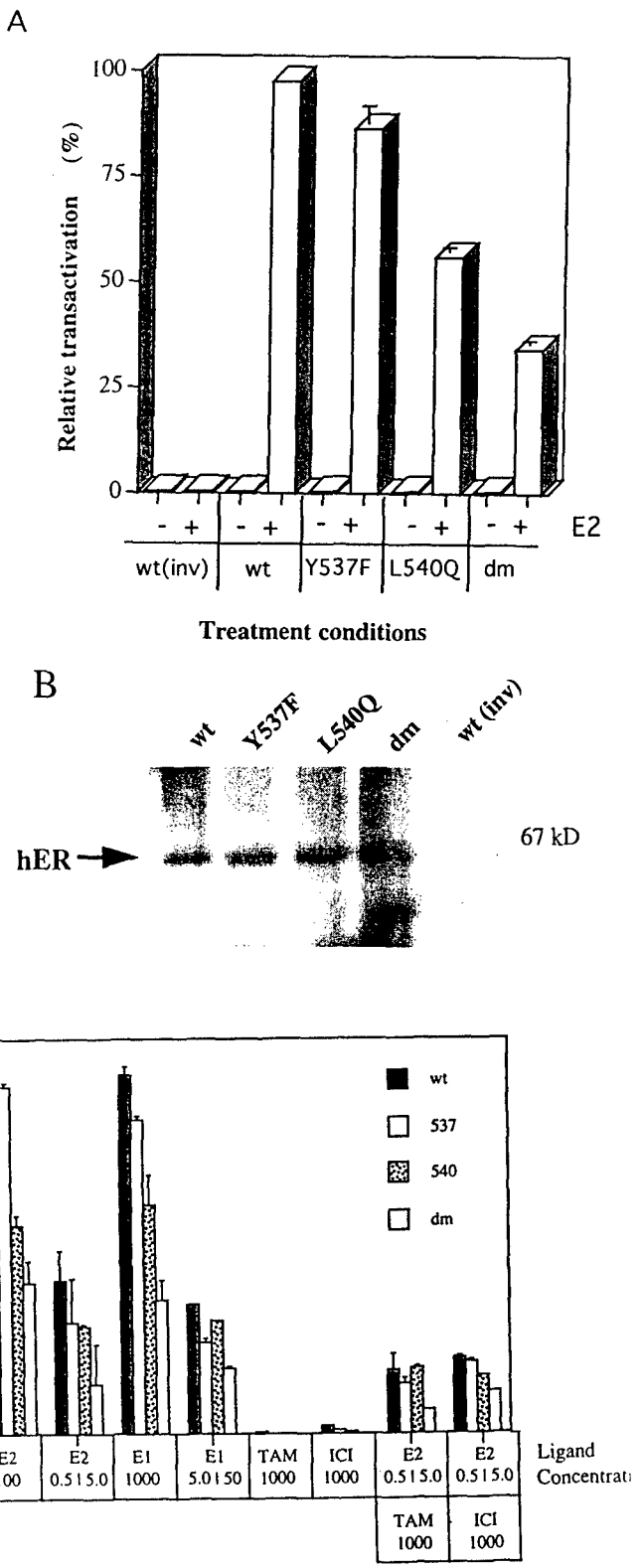
A gel shift assay with using in-vitro translated hER and hER fragments shows that the presence of the ligand binding domain in the C-terminal end of the receptor is required for inhibition by the phosphopeptide Yp-11. The first lane contains Sf9 expressed wild type hER as a control. The remaining lanes contain in-vitro translated protein extracts - WT, full length hER; N, amino terminal hER including regions A-D (see figure 1); C, carboxy terminal hER including regions C-F. Lane 2 and 3 contain wt and N hER with an anti-hER antibody raised against an epitope within the A/B region. Arrows indicate the specific hERs bound to ERE and the location of non specific (NS) bands.

Figure 6. Transcriptional Activation of WT and Mutant hER in Yeast

A, The yeast strains containing either the wild type or indicated mutant hER and the ERE containing reporter were grown for 18 hr in the presence or absence of 10 nM estradiol at 30 C. Following lysis, transcriptional activity of hER was measured by the β-galactosidase assay and are shown as percent of maximal response, expressed in Miller units. The data shown are the means +/- SEM for 3-4 samples from three separate experiments.

B, Yeast cells prepared as above were lysed in SDS sample buffer, boiled for 5 min., and subjected to western blot analysis with anti-hER antibody. A representative result from 3 experiments is shown.

C, The yeast cells expressing the indicated hER and reporter plasmid YRPE2 were grown in medium containing indicated ligand at 30 C for 16 h. Abilities of the antiestrogens tamoxifen citrate (TAM) and ICI 182, 780 (ICI) to block estradiol-mediated transactivation of the hERs were measured at the concentration of estradiol (E2) corresponding to a half maximal response.



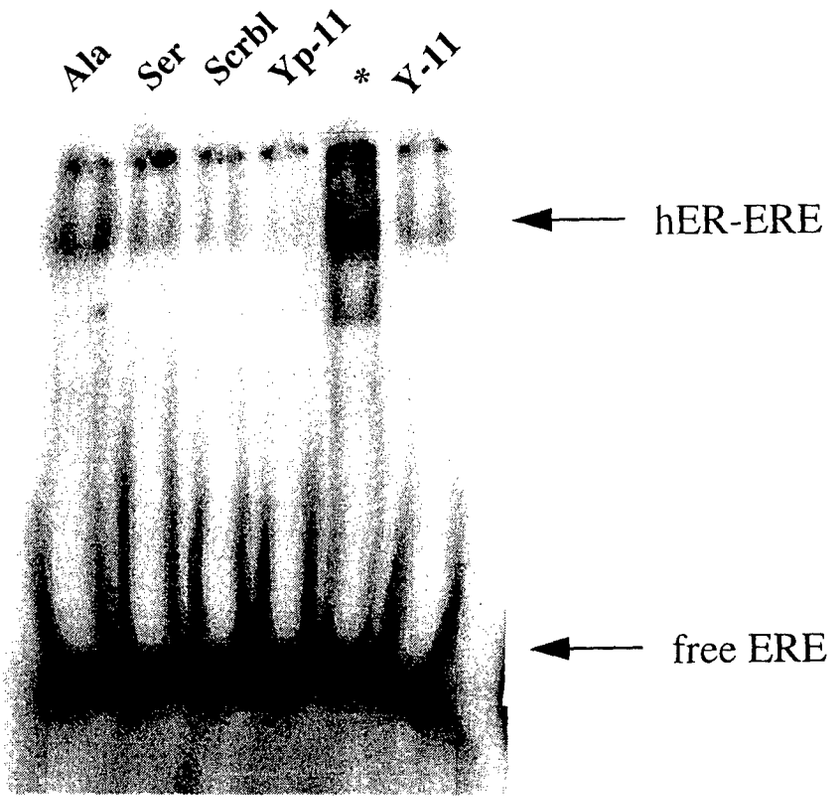


Figure 7. Substitutions for Phosphotyrosine Abolish Inhibitory Effects of Peptide.

A gel shift assay with various phosphotyrsoine substituted peptides. Refer to figure 1b for sequences of these peptides. Lane 5 (*) contains wt hER incubated with reticulocyte lysate produced hER fragment A-D (fig 1 C).

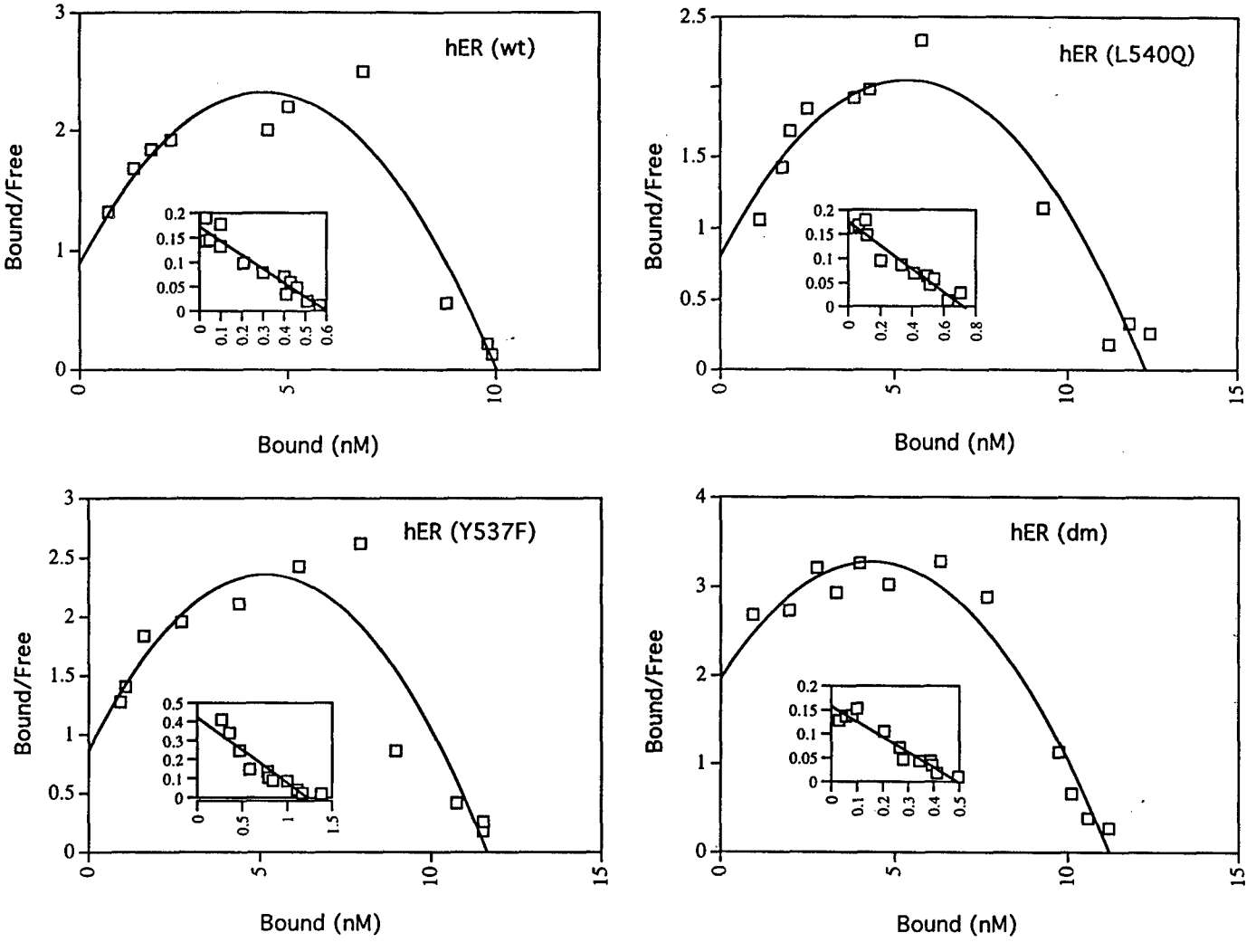


Figure 8 Saturation Analyses of the [3H] estradiol binding of the mutant and wt hERs.

Aliquots of extracts containing 0.8-12 nM of receptors were incubated for 16 hr on ice with increasing concentration of [3H] estradiol. Specific ligand binding was determined by subtraction of non-specific binding in the presence of a 200-fold excess of radio inert estradiol from the total binding observed. The data were analyzed by the methods of Scatchard and Hill. The relative affinities were estimated from fitted saturation plots and did not vary between wt and mutants more than 25% for any given measurement. Maximum Hill coefficients ranged from 1.2-1.6 +/- 0.2. A representative Scatchard plot for each mutant is shown at both high and low (insert) concentrations

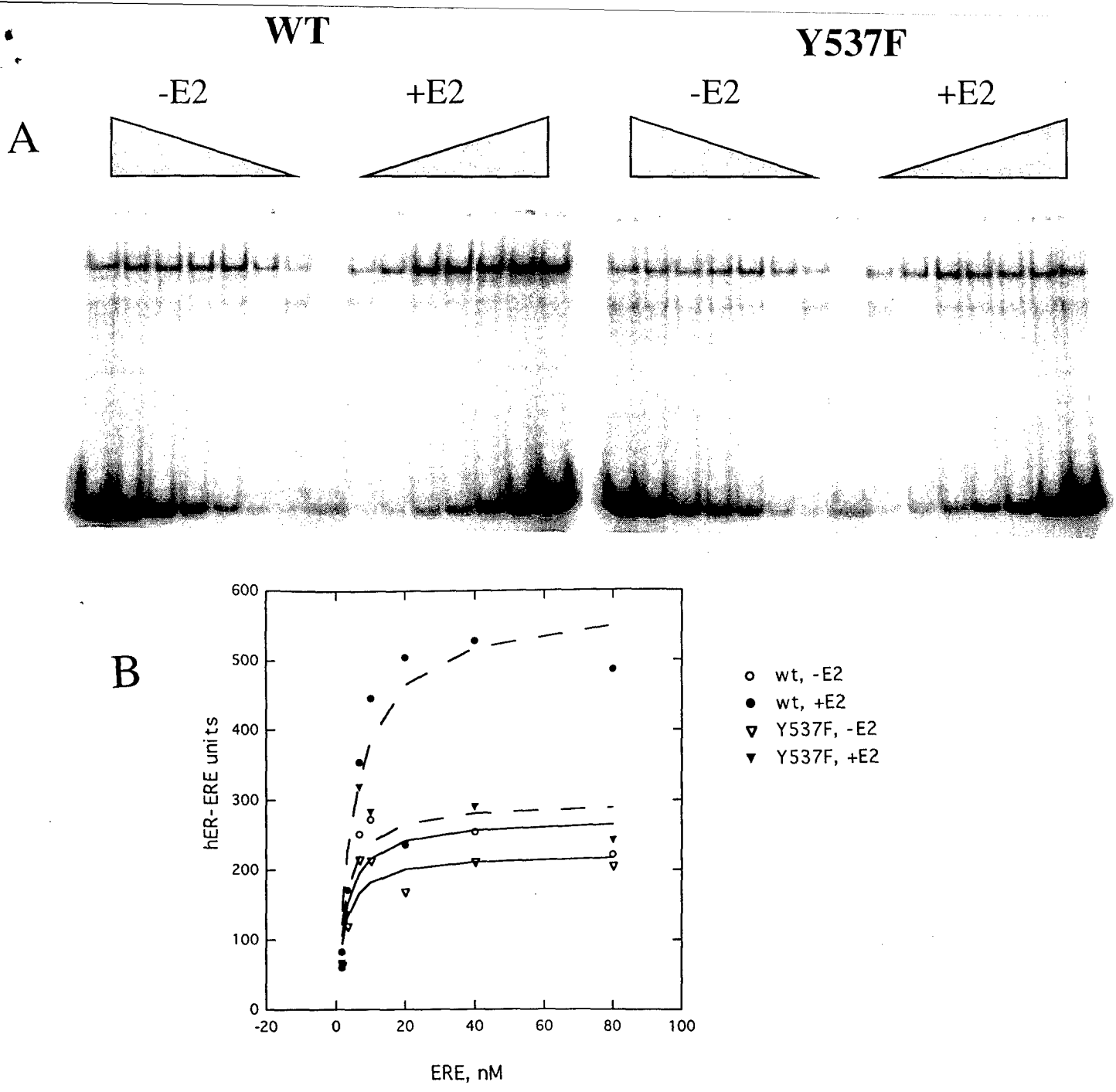


Figure 9. The DNA Binding Capacity of the hER is Compromised in Y537F.

A, The wt and Y537F hER were incubated overnight on ice in both the absence and presence of 10 nM estradiol before incubation with increasing concentrations of ^{32}P-labeled ERE probe. The wt hER is shown on the left gel and the Y537F is shown on the right gel. In each gel the left half is minus estradiol, and the right half is with the hormone.

B, The same data in A, plotted as a function of ERE added in nM. The ordinate is relative units as measured on the PhosphorImager from equal exposure time for each gel. The relative affinities of wt and Y537F hER do not vary more than 2 fold from experiment to experiment. A representative graph from three separate experiments is shown.

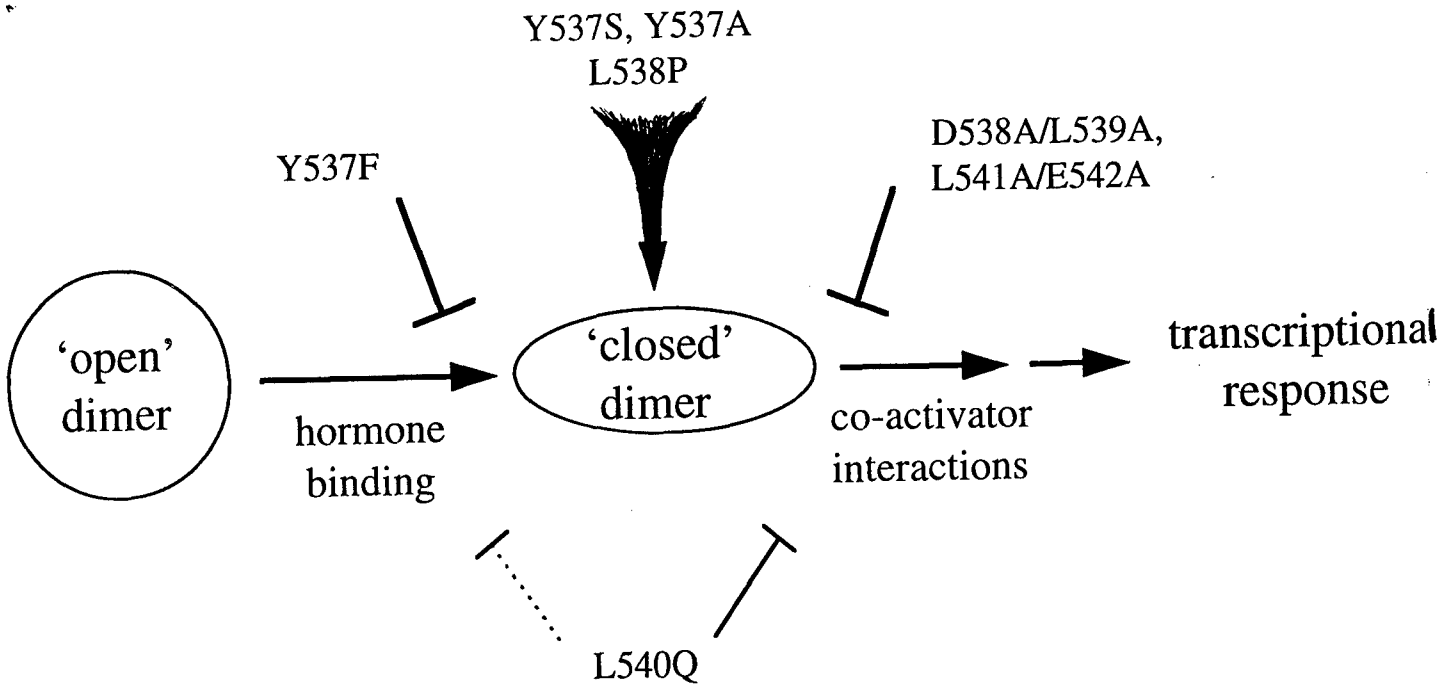


Figure 10. A model illustrating the effects of various helix 12-region mutations on a hypothetical pathway of hER activation.

The activation of the dimeric wild type hER proceeds through a hormone binding event which results in a conformational change of the helix 12 region and the conversion from an open to a closed domain. The indicated mutations are proposed to affect either the formation of the closed complex (left half reaction), or the interaction with coactivators (right half reaction). The mutations shown are discussed in the text and are taken from the literature (10-14, and references therein).

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